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Nucleotide Base Variation of Blast Disease Resistance Gene *Pi33* in Rice Selected Broad Genetic Background

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Rice is one of the most important crops for human beings, thus increasing productivity are continually persecuted. Blast disease can reduce the rate of productivity of rice cultivation. Therefore, the program of blast disease-resistant varieties needs to do effectively. One of broad-spectrum blast disease-resistant gene is *Pi33*. This study was aimed to identify the variation in the sequence of nucleotide bases of *Pi33* gene in five interspesific lines which derived from Bio46 (IR64/*Oryza rufipogon*) and CT13432 crossing. DNA of five rice lines were amplified using the spesific primer for *Pi33*, G1010. Amplification results purified through Exonuclease 1 and Shrimp Alkaline Phosphatase protocols. Labelling using fluorescent dyes done before sequencing nucleotide base using CEQ8000 instrument. The results showed that lines number 28 showed introgesion of the three control parent genome (subspecies of Indica, subspecies of Japonica, and *O. rufipogon*) while the Lines number 79, 136, and 143 were identical to Indica genome. Strain number 195 was identical to Japonica genome. These broad genetic background lines promise as durable performance to attack the expansion of the dynamic nature of the pathogen to blast. The result of ortholog sequence analysis found conserved nucleotide base sequence (CAGCAGCC) which involved in heterotrimeric G-protein group. This protein has role as plant receptor for recognizing pathogen elicitor in interaction of rice and blast pathogen.

Key words: nucleotide base, blast disease, *Pi33* gene, rice

INTRODUCTION

Blast disease is one of the significant diseases found in rice due to pathogenic fungi, *Pyricularia grisea*. The disease reduce world rice production level by 30-50% (Baker *et al*. 1997; Scardaci *et al*. 1997). The blast pathogen is dynamic due to rapid adaptation to host condition. Control of Blast disease is carried out using resistant rice variety; it is regarded as one of the most effective method to control the spread compare to fungicide application which give negative impacts to the environment. Therefore, development of rice blast-resistant program becomes very essential. The new blast resistance rice lines breeding was carried out using molecular marker selection approach. Indonesian Centre Agriculture and Biotechnology Genetic Resources, R & D Institute (ICABIOGRAD/BB-Biogen) has generated BIO46 the wide-spectrum rice lines resistant to several blast races. BIO46 line is a double haploid line derived from the parents IR64 and the wild rice species *Oryza rufipogon* which contains of blast-resistant genes, *Pir4* and *Pir7* (Utami *et al*. 2008). Furthermore, Centro International de-Agriculture Tropica (CIAT) also has generated a multigenic rice lines with potentials wide-spectrum resistance to blast disease. This line was named CT13432, contained three of blast resistance genes, *Pi1*, *Pi2,* and *Pi33* (Tharreau 2007). To obtain the double haploid rice lines which have a several blast resistant genes, BIO46 was crossed to CT13432.

One of the blast pathogenic avirulent gene characterized was ACE1 which give specific reactions to *Pi33*, a blast resistance gene in rice plant (Berruyer *et al*. 2003). Interaction between the blast resistance gene and pathogenic *avr* gene, the gene which attack ability contribute of the pathogen could be identified for better understanding of gene to gene interaction between rice and blast pathogen. In order to determine the specification of resistance spectrum in various selected rice lines, an identification of nucleotide variation of blast-resistant gene, *Pi33* was necessary to be carried out. Based on this identification, parent's introgression on selected rice lines progenies could be related to their blast resistance performance.

MATERIALS AND METHODS

Blast Resistance Phenotype Evaluation of the Selected Lines. The selected lines from crossing between BIO46-CT13432 and Kencana Bali variety as a control plant were planted in greenhouse and fields. Greenhouse planting using pot (40 x 29 x 7 cm) filled with compost media. Every row in the pot, 10-15 seeds of each line was planted. Soil humidity was maintained through watering and

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procurement of nutritional solution every week. Nitrogen fertilizer used 8.6 g per pot and was applied on 10, 3, and 1 day before inoculation to increase the sensitivity plant to blast. Blast isolates were used for this test were grown in Rice Flour Medium at a temperature of 25 °C with 12 hours of photoperiodic cycle. After 3-4 weeks old (4-5 leaves) of each rice lines were inoculated by spraying with 30 ml of conidia suspension (50,000 conidia/ml) and 0.5% gelatin. Inoculated plants are then incubated in the dew chamber at a temperature of 24 °C and 95% humidity for 16 hours.

Three blast races were used in this phenotype evaluation, each contained *ACE1* gene with PH14 genotype for Race173, CM28 for Race 063, and Guy11 for Race 101. To confirm the blast performance of selected rice lines were planted in blast endemic location in the field test (in Sukabumi), thus exposing them to natural blast infection. To confirm the level of blast resistance, the selected rice lines were grown in the blast disease endemic field (in Sukabumi) to allow direct interaction with a natural blast pathogen in the field. Blast score (damage level) of every plants was assessed in accordance to the System Evaluation Standard (SES) (IRRI 1996). The blast scoring was carried out on vegetative stage (± 1 month old), for both green house and field test.

DNA Isolation of Selected Lines (Dellaporta *et al.* 1983; Sambrook & Russel 1989). Half gram of rice plant leaves from the selected rice lines (No. 28, 79, 136, 143, and 195) as lines contained *Pi33* gene was used as DNA source. A total of 700 µl of extraction buffer (NaCl, Tris-HCl, EDTA, and SDS) was added and incubate in 65 °C (15 min). Then, 700 µl of chloroform was added and the next suspension was centrifuged in 12,9 G (5 min). Supernatant was extracted and added with 50 µl of ammonium acetate and 800 µl of absolute ethanol, and then centrifuged (12,9 G for 5 minutes). The DNA obtained during the purification stage was typically in the form of liquid DNA solution of which the concentration must be increased and settled through centrifuge. DNA concentration was obtained through absolute ethanol precipitation in saturated condition (Na^+). White pellets were washed with 500 µl of ethanol 70% and dried in the oven (± 50 °C) for 15 minutes. The dried pellets were dissolved in 50 µl of TE solution (Tris-EDTA).

Amplification of *Pi33* Gene in the Selected Rice Lines. Five DNA samples of the selected rice lines are amplified using G1010 primer (Berruyer *et al.* 2003), (F/R: 5'-CCAAGTATTCTAGCTCGCTGTC-3'/5'-TGCTAGAGA TTTGAGAAGATGG-3'). Optimization of PCR was carried out with total volume of 20 µl, which consists of 3.2 µl of dH_2O, 2.5 µl 10 x PCR buffer, 3 µl of 10 mM primer, 1 µl of 200 µM dNTP, 4 µl of 5 x GC-rich, 0.3 µl of DNA enzyme Taq polymerase, and 6 µl of DNA sample. PCR process consisted of four stages: early denaturalization at a temperature of 94 °C for 1 minute, annealing at a temperature of 50 °C for 1 minute and primer extension at a temperature of 72 °C for 2 minutes. All four stages were carried out for 35 cycles, continued with 2 minutes of storage at a temperature of 34 °C to prepare the sample for the last extension step. The extension step was carried out at a temperature of 72 °C for 5 minutes and the last stage, stored at a temperature of 15 °C. PCR product was then purified using EXO-SAP purification.

The lambda DNA's concentration was used to calculate DNA sample's concentration by comparing the area or width of electrophoresis band. The concentration of lambda DNA used varies between 25, 50, 100, and 150 ng/µl. The purity of result of DNA isolation on all five selected rice lines were measured using spectrophotometer at wave length of 260/280 nm.

Analysis of BLAST Sequence and Phylogeny Tree Construction. Nucleotide were analysis by using BioEdit software version 7.0.9.0. Alignment sequence was carried out with Indica (Gramene) genome and Japonica (TIGR Japonica) genome. The BLAST Sequence analysis consists of offline and online analysis as well as profiling analysis on the nucleotide base sequence and analysis on genetic closeness of all five selected rice lines to the *Pi33* gene. Online sequence analysis was carried out on browser genome rice from subspecies groups of indica, japonica, and *O. rufipogon* species.

The finding of conserved area was carried out using Greenphyl genome in the Ortholog part to identify the function of the conserved gene's motive. Phylogenetic tree construction of each selected strain for *Pi33* sequences gene based on Neighbor-Joining/UPGMA (Unweighted Pair Group Method Arithmetic Mean) method version 3.6a2.1.

RESULTS

Blast Resistance Phenotype Evaluation on Selected Rice Lines. All leaves of five selected rice lines showed area of blast infection. The resistant lines (R), have score varies between 0-3 with the infected lesions area less than or same as 5% of total leave length area, whereas the susceptible strain (S) has a score of 4-9 with the infected lesion area more than 5% of total leave length area. The result of resistance level test in Table 1 showed that each rice line has different response to different blast races with different *ACE1-avr* gene. It might be each line gave different pathosystem in basic of interaction between *resistance* gene (*Pi33*) in rice lines and different *ACE1-avr* gene in blast races. These difference pathosystem interaction performances probably were related with the difference sequence profile particularly on their blast genes resistance, *Pi33*.

The blast susceptible variety control, Kencana Bali was shown susceptible performance for all three blast races. It was indicated that the inoculation system is exertion well as a blast control system screening.

Among the five selected rice line, lines number 28 and 195 were give resistant respond to three blast races. These two lines indicated has blast resistance gene which could resolve the aggression of the pathogen which one of this performance contributed by the different *ACE1-avr* gene of blast races. Lines number 79 and 136 were showed the resistant performance only to Race 101, which has *Guy11*

Table 1. Response resistance of five selected Lines against different races of blast

Rice lines	Testing in the green house (blast race)			Testing in the field
	173 (PH14)	063 (CM28)	101 (Guy11)	
28	R	R	R	R
79	S	S	R	R
136	S	R	S	S
143	R	S	R	R
195	R	R	R	R
K.Bali (susceptible control)	S	S	S	S

R (resistance): score 0-3; S (susceptible): score 4-9.

genotype of *ACE1-avr* gene and Race 063, which has *CM28* genotype of *ACE1-avr* gene, respectively. Lines number 143 was showed the resistance performance to Race 173 and Race 101.

Blast evaluation of five selected lines in the field test, the inoculation of blast pathogen to rice lines test was occurred naturally, so the interaction between R genes with *avr* genes was unknown. However, blast resistance testing for selected rice lines in the field, can indicate the blast resistance level of the lines in the face of the blast pathogen genetic diversity that develops in the testing field.

DNA Isolated and PCR Analysis of *Pi33* Gene of Selected Rice Lines. The electrophoresis of isolated DNA uses comparison control in the form of lambda DNA with known concentration (Figure 1a). The isolated DNA's electrophoresis from all five selected rice lines (Figure 1) showed that the concentration of DNA samples were comparable to lambda DNA, equal to λ50 control. The result of quantification of isolated DNA from all five selected rice lines were showed purity value between 1.6-1.8 (Table 2). Better qualities of DNA were characterized by non-degraded DNA, obvious from the lack of DNA smear band on the agarosa gel.

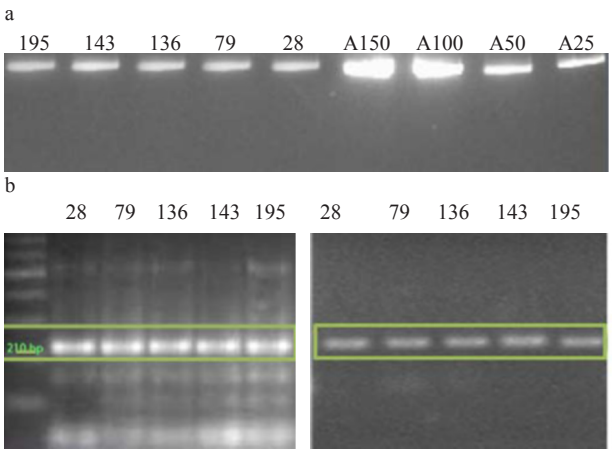

Figure 1. a. The quality of DNA isolated of each rice lines; b. the results of PCR amplification and EXO-SAP purification resulted. Sample order from left to right: 28, 79, 136, 143, and 195.

Amplification of *Pi33* gene on all five selected rice lines: 28, 79, 136, 143, and 195 used PCR-based on gene specific molecular marker revealed 210 bp (Figure 1b). These PCR products were continued on EXO-SAP purification that showed clearly specific DNA band (Figure

Table 2. Matrix of genetic proximity of five selected lines comparing to the genome control of their parents

	28	79	136	143	195	*O. indica*	*O. rufipogon*	*O. japonica*
28	-	2.34	1.65	0.37	0.71	1.71	1.80	1.91
79		-	1.85	2.17	2.28	1.37	2.64	1.66
136			-	1.82	1.76	1.75	2.20	2.02
143				-	0.72	1.37	2.09	1.67
195					-	1.87	2.23	1.79
O. indica						-	1.30	0.28
O. rufipogon							-	1.55
O. japonica								-

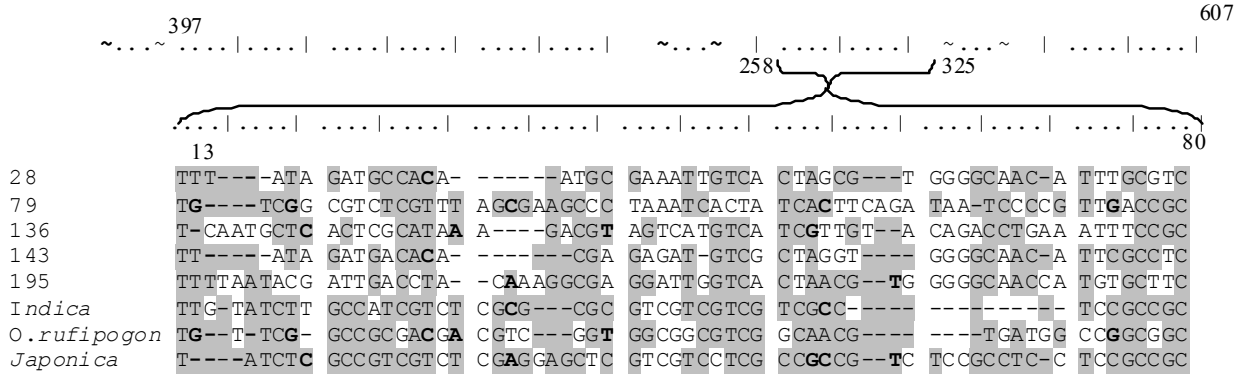

Figure 2. Part profiling nucleotide base sequence analysis of five selected lines comparing all genome rice subspecies. The best significant homolog was on base position 258-325 of the total sequence 397-607 of the alignment analysis. Nucleotide bases in gray areas and some in bold were indicated the homology alignment with the genomes of the three sub-species of rice, *Indica, Japonica,* and *Oryza rufipogon* as a genome background parents.

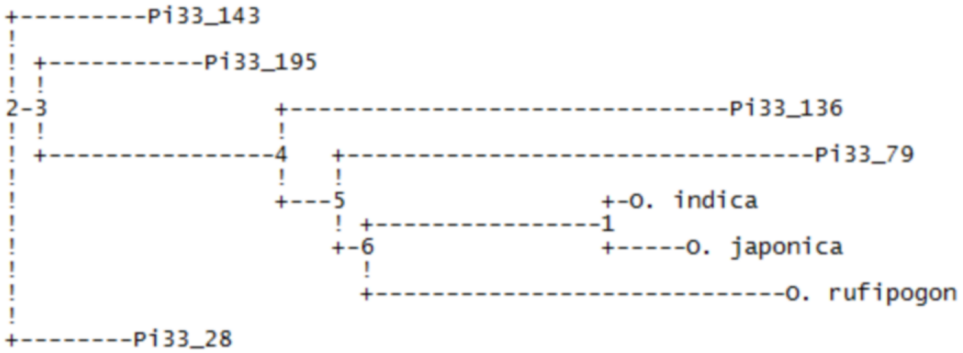

Figure 3. The phylogenetic tree of five selected lines comparing to the genomic control on part of *Pi33* gene sequence.

1b on right side). The DNA samples seem still contains protein contaminant (Table 2), with the purity value of less than 1.8. However, the isolated DNA could be amplified directly using PCR technique, as this technique does not require DNA with high purity and only needs DNA volume in micro liter unit. However, to ensure that the amount of DNA to be amplified with PCR has even concentration, the DNA concentration value obtained has to be uniformed through dilution with dH_2O into 10 ng/µl of concentration level.

BLAST Sequence Analysis. The profiling analysis of nucleotide base sequence was based on the introgression of the parents genome (CT13432/ Japonica, Bio46/Indica (IR64)-*O. rufipogon*) and the identification of conserved sequence as shown in Figure 2.

Matrix of genetic similarity in Table 2 and Figure 3 showed that among all five selected rice lines, the lines with most genetic similarity to Indica, Japonica subspecies and *O. rufipogon* genome is lines 28. This line has the introgression from all three parents' genome control. It is reasonable to state that there has been an introgression of nucleotide bases in all three controls on lines 28. Meanwhile, line no. 79, 136, and 143 have genetic similarity to Indica subspecies (BIO46), whereas line no. 195 has genetic similarity to Japonica subspecies (CT13432).

The result of phylogenetic tree of each selected rice line contained the *Pi33* gene target based on its nucleotide base sequence uses the Neighbor-Joining/UPGMA (Unweighted Pair Group Method Arithmetic Mean) method version 3.6a2.1 on Tassel Software program, comparing to the Subspecies rice genomic sequence as a control (Figure 3). While the genetic distance of each selected rice line was shown in the matrix of genetic proximity on Table 2.

DISCUSSION

The genetic materials used in this research were the five selected lines, progenies of crossing between BIO46 and CT13432, which contained the blast-resistance gene, *Pi33* detected by using the specific marker for the *Pi33* gene (G1010). Furthermore, the five lines were also as selected lines for several agronomic characteristics such as flowering age, plant vigor, number of productive tiller and unhulled rice color. This phenotype diversities were important factors in blast-resistant line development of promising rice lines.

Further, The resistance of rice variety to blast disease is also influenced by the genetic diversity of the pathogen growth taking place in the field. While the pathogen attack ability heavily influenced by the virulence factors possessed the certain race of the blast pathogen. So it occurs the interaction between the blast resistance gene in a rice line (*Pi33*) with a virulence gene (*avr gene*) that is owned by the blast pathogen. One of the *avr* genes which already characterized was *ACE1-avr gene*, as a poly keto syntetase (PKS/NRPS) protein coded. This protein was contained of 4035 amino acids and has function as catalyze on pathogen penetration to their host plant. (Bohnert *et al.* 2004). *Avr* gene *ACE1* has two copies genes which showed the different genotype of blast isolate. First, *Guy11* genotype has a *ACE1* copy gene in chromosome 1 of the blast genome. This isolate is likely to be avirulent. Second, *CM28* genotype has a *ACE1* copy gene in chromosome 6, this isolate is virulent. Another genotype was definite if the isolate has two copies of *ACE1* gene, *Guy11,* and *CM28*, this isolate as a *PH14* genotype (Couch *et al*. 2005). So, the three genotypes, *Guy11, PH14,* and *CM28* of *ACE1* correspond to different genotypes of blast isolates. The distribution of *ACE1* was assessed using allele-specific PCR (Fudal 2004).

Several dominant blast pathogen races on the field with different avr gene are: Race 173, contained *PH14 avr* gene, Race 063, contained *CM28 avr* gene, and race 101, contained *Guy11 avr* gene (Santoso *et al*. 2007). These dominant blast races with different *ACE1*-genotype were necessary used in blast resistance screening system as represent the dynamic of genetic diversity blast population.

Result of tests on the five selected lines against the three dominant races of blast pathogen showed that the selected rice lines have different resistant respond to different blast disease, particularly on greenhouse screening results (Table 1). This is possible due to the different response the R gene and avr gene on interaction between the resistance genes in rice lines to *avr* genes in blast races. In contrast, in the field test, the inoculation of blast pathogen to rice lines test was occurred naturally, so the interaction between R genes with avr genes was unknown. However, blast resistance testing for selected rice lines in the field, can indicate the blast resistance level of *Pi33* gene in the face of the blast pathogen genetic diversity that develops in the testing field.

Such use of primer is based on previous research by Kurata (1994). However, the result of observation on PCR product (Figure 1) shows a less specific band pattern at base length of 210 bp. This phenomena is likely caused by the lack of optimal annealing temperature. This is shown by the agarose gel which has more than 1 band uneven in thickness at base length of 210 bp. The next stage after the procurement of PCR product would be sample purification from primer and free dNTP using Exonuclease 1 and Shrimp Alkaline Phospatase. The success of this stage is shown by the appearance of thick band pattern at base length of 210 bp.

The genetic position and locus of the *Pi33* gene which acts as a control is determined based on the result of mapping of rice genome and is selected using the MAP Kinase Putative Function Search Tool on Rice Genome Annotation (http://rice.plantbiology.msu.edu/index.shtml). The *Pi33* gene is located at a position of 3,306-3,309 Mbp inside the LOC-Oso8g32600 locus. Offline sequence analysis is carried out using BioEdit V7.0.9 program (Hall 1999). The purpose of this analysis is to scan the result of *Pi33* gene nucleotide base sequence in each selected strain against the *Pi33* gene control. Meanwhile, the purpose of online sequence analysis is to compare the nucleotide base sequence of each analyzed strain with the elder rice genome control. The profiling analysis has the purpose to identify the introgression type of each selected strain from its elders. Through the analysis, possibly to identify that all five selected rice lines of which the nucleotide base sequences were analyzed against *Pi33* gene have conserved base motive of CAGCAGCC, included as a group of G-heterotrimeric protein which serves as the plant's receptor for identifying pathogenic elicitor in the interaction between rice plants and blast pathogen (http://greenphyl.cirad.fr). The variation of nucleotide base in each five selected rice lines showed that the variations was caused by the introgression of its parents genome from indica and japonica subspecies and *O. rufipogon* species. Based on this sequence diversity one can analysis possibility to detect rice lines with have broad genetic background. Subsequently, based on this results could facilitated to define the new rice lines with a durable performance to attack the expansion of the dynamic nature of the pathogen to blast.

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